The Amino-Terminal Tail of Glycogen Phosphorylase Is a Switch for Controlling Phosphorylase Conformation, Activation, and Response to Ligands†

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ABSTRACT: Glycogen phosphorylase is a muscle enzyme which metabolizes glycogen, producing glucose-1-phosphate, which can be used for the production of ATP. Phosphorylase activity is regulated by phosphorylation/dephosphorylation, and by the allosteric binding of numerous effectors. In this work, we have studied 10 site-directed mutants of glycogen phosphorylase (GP) in its amino-terminal regulatory region to characterize any changes that the mutations may have made on its structure or function. All of the GP mutants had normal levels of activity in the presence of the allosteric activator AMP. Some of the mutants were observed to have altered AMP-binding characteristics, however. R16A and R16E were activated at very low AMP concentration and crystallized at low temperature, like the phosphorylated form of GP, phosphorylase *a*, and unlike the dephospho-form, phosphorylase *b*. This indicates that even without phosphorylation, the structures of these mutants are more like phosphorylase *a* than phosphorylase *b*. These mutants were also very poorly phosphorylated in the presence of the inhibitor glucose, while phosphorylase *b* was phosphorylated normally with this inhibitor present. In contrast to R16A and R16E, four other mutants behaved like phosphorylase *b* after phosphorylation. R69E was only partially activated by phosphorylation, and I13G, R43E, and R43E/R69E were completely inactive after phosphorylation. We propose a model for the many functions of the amino terminus to explain the many varied effects of these mutations.

Glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) (GP)[1] is found in many different mammalian tissues, such as muscle, liver, and brain, and similar forms are also found in yeast, plants, and bacteria (*1*). The most extensively studied form is rabbit muscle GP. GP catalyzes the breakdown of the storage polysaccharide glycogen to produce glucose-1-phosphate (G1P), which can then be used for the production of ATP. Glycogen phosphorylase is therefore a very important component of the carbohydrate metabolic pathway in muscle cells.

The regulation of glycogen phosphorylase activity is quite complex. Phosphorylase *b* (phos. *b*) is the inactive form of the enzyme. It can be activated by the binding of AMP (*2*, *3*), or by phosphorylation on residue Ser 14 by phosphorylase kinase (PhK) (*4*, *5*). Phosphorylation converts phos. *b* to phosphorylase *a* (phos. *a*), which is active in the absence of AMP. Once activated, GP must be inactivated when glycogen catabolism is no longer needed. Several factors work to inhibit GP activity. These include dephosphorylation of phos. *a* by protein phosphatase-1 to produce phos. *b* (*6*, *7*), dissociation of AMP, and the binding of many different inhibitory molecules. Glucose-6-phosphate (G6P), glucose, and caffeine are common GP inhibitors (*1*).

The functional form of GP is a homodimer of 842-residue subunits (*8*, *9*). This quaternary structure allows for allosteric binding, as there are two binding sites for each effector per enzyme. The allosteric binding of different effectors produces different conformations of GP, either active or inactive. Similar active and inactive conformations are produced by phosphorylation and dephosphorylation (*10*, *11*). The structures of phos. *b* and *a* have been known for over 20 years (*12*−*14*). Activation of GP produces a rotation of the subunits with respect to each other and causes them to be pulled closer together on one side, opening up the active site on the other side. The two methods of activation (AMP binding or phosphorylation) produce a similar conformation, with the only major difference being the structure of the amino terminus (*10*, *15*).

The N-terminal 20 residues, containing the phosphorylation site, form a sort of extended tail, away from the folded core of the enzyme. This tail, containing numerous basic residues, has a disordered structure in phos. *b* and rests on the surface of the enzyme in an area of negative charge (*16*). Upon phosphorylation of the Ser 14, the N-terminus forms a 3_{10} helix and rotates about 90° so that it binds at the subunit

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[1] Abbreviations: PhK, phosphorylase kinase; phos. *b*, phosphorylase *b*; phos. *a*, phosphorylase *a*; GP, glycogen phosphorylase; RMGP, rabbit muscle glycogen phosphorylase; YGP, yeast glycogen phosphorylase; TCA, trichloroacetic acid; AMP, adenosine 5′-monophosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; 40/5 buffer, 40 mM β-glycerophosphate/5 mM DTT, pH 6.8.

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interface (*10*, *11*). There, the serine phosphate is complexed to a number of basic amino acids, Arg 69 and Arg 43′ (the prime denotes a residue from the opposite subunit), through salt-bridge interactions, which are thought to hold the N-terminus in place. This movement of the N-terminus does not occur in AMP-activated phos. *b* (*11*, *17*), yet a similar conformation is produced in the core of the enzyme (*15*).

The different conformations of GP are very important for its regulation and activity. These conformations can be considered within the Monod model for allosteric enzymes (*18*). In their model, the "T state" of an allosteric enzyme has low activity and low affinity for substrates and effectors, while the "R state" has high activity and high affinity for substrates and effectors. A given effector will promote or stabilize either the R state or the T state, which can be seen by its effect on the activity of the enzyme. These may not be the only conformations in which the protein can exist, however. Phosphorylase demonstrates that the R and T states are not discrete, all-or-nothing conformations. The conformation produced by each of GP's effectors can be different depending on whether it is bound to phos. *a* or *b* (*19*−*21*), and GP can be phosphorylated or dephosphorylated differently, depending on which, if any, effectors are bound (*22*−*24*). The structures of the R and T states of GP have been studied extensively (*10*, *11*, *25*). There are many questions which remain, however, about how the different conformations are produced, i.e., what tertiary, secondary, and primary structural elements are involved in contributing to or stabilizing certain conformations.

Even though GP is a large enzyme, it is possible that individual amino acid residues may affect its conformation. To study this, we have characterized 10 site-directed mutants of GP, looking for changes in their biochemical properties, which may indicate changes in conformation. Three residues in the N-terminus were mutated, Lys 11, Ile 13, and Arg 16, along with three residues that interact with the N-terminus in either the phospho- or the dephospho-state, Arg 43, Arg 69, and Glu 501 (*26*). Several of these mutants had different AMP-binding characteristics, an indicator of conformational state. Some could also be forced by different GP effectors into conformations which could not be phosphorylated by the kinase core of the PhK catalytic subunit, γ(1−300). Others could not be activated by phosphorylation. These many varied results from altering individual residues demonstrate the importance of specific amino acids for phosphorylase structure and activity, primarily by affecting the structure and position of the amino terminus.

EXPERIMENTAL PROCEDURES

Materials. The plasmids RMGP-Nde53 and RMGP-pTacTac, containing the cDNA of rabbit skeletal muscle phosphorylase, along with mutagenesis primers for mutants R16A, R69K, and E501A, were generous gifts of Dr. Robert Fletterick, UCSF. All other mutagenesis and DNA sequencing primers were synthesized in the Iowa State University DNA Facility on an Applied Biosystems 3948 Nucleic Acid Synthesis and Purification System. *E. coli* 25A6 cells were kindly provided by Genentech, South San Francisco, CA. Oyster glycogen type II was purchased from Sigma, St. Louis, MO. Chelating Sepharose Fast Flow and DEAE-Sepharose Fast Flow were from Amersham Pharmacia, Piscataway, NJ.

Mutagenesis, Protein Expression, and Purification. Mutagenesis was performed as described (*26*). Expression of recombinant GP and mutant forms was carried out, as described (*26*), in the pTacTac expression vector in *E. coli* strain 25A6, according to the method developed by Browner et al. (*27*). Purification of recombinant wild-type phosphorylase and mutants was carried out by chromatography on a Cu^{2+}-affinity column, followed by a DEAE-Sepharose column, as described (*26*, *28*). Final samples were concentrated, dialyzed into 40 mM β-glycerophosphate/5 mM DTT, pH 6.8 (40/5 buffer), and stored at 4 °C. Phosphorylase purified in this manner was 95% pure, as judged by SDS−PAGE. Phosphorylase *b* was purified from rabbit skeletal muscle (*29*) and recrystallized at least 3 times. It was then dialyzed into 50% 40/5 buffer, pH 6.8/50% glycerol and stored at −20 °C. Concentrations of phosphorylase samples were determined spectrophotometrically at 280 nm, using the extinction coefficient of 1.32 cm^2/mg of phosphorylase (*30*, *31*). Phosphorylase kinase catalytic subunit, γ(1−300), was expressed and purified as previously described (*32*) and stored in 50% glycerol at −20 °C.

AMP Activation Assays; K_m, n_H *Determination*. AMP activation assays were performed by measuring phosphorylase activity spectrophotometrically using the method of Krebs et al. (*22*), with increasing concentrations of AMP, from 0 to 1 mM. In some assays, the phosphorylase effectors glucose and caffeine were also included, at 10 and 1 mM, respectively. K_m and the Hill coefficient (n_H) for AMP were determined from Hill plots of AMP activation assays performed in duplicate. Values reported for K_m and n_H are the mean ± SD for values derived from three Hill plots, except where otherwise stated.

Phosphorylation Assays. Time courses of phosphorylation were performed at 2 mg/mL phosphorylase, 1 mM ATP, 10 mM $MgCl_2$, 2 mM DTT, in 50 mM PIPES/50 mM Tris, pH 8.2. Reactions were incubated at 30 °C for 30 min with γ-(1−300) (0.05−0.1 μg/mL), or with buffer for blanks. Progress of reactions was monitored either by the incorporation of ^{32}P from [γ-^{32}P]ATP or by the increase in phosphorylase activity without AMP. For the isotopic assays, 10 μL aliquots were removed from the reaction at various times and spotted on Whatman 31ET paper. Papers were washed for 30 min each in 10% trichloroacetate (TCA)/1% pyrophosphate, 5% TCA/1% pyrophosphate, and 5% TCA, and then dried. ^{32}P incorporation was detected by liquid scintillation counting, and blank values were subtracted from all data. For measuring the increase in phosphorylase activity, 10 μL aliquots were removed at various times and diluted to 20 μg/mL GP in cold 40/5 buffer, pH 6.8. Phosphorylase activity was then assayed at 10 μg/mL in the absence or presence of 1 mM AMP, after the method of Krebs et al. (*22*). Some phosphorylation assays with R16A and R16E included phosphorylase effectors. In these assays, phos. *b*, R16A, and R16E were used at 2 mg/mL with 0.2 μg/mL γ(1−300). When used, G6P was 5 mM, glucose was 10 mM, caffeine was 1 mM, and AMP was 300 μM, unless otherwise noted.

IC_{50} experiments for glucose inhibition of phosphorylation were similarly performed at 2 mg/mL phosphorylase, 0.2 μg/mL γ(1−300), with increasing concentrations of glucose, from 0 to 10 mM for R16A and R16E, and from 0 to 1 M for phos. *b*. Phosphorylase was preincubated with glucose

Table 1: Sequence Changes of GP Mutants in Comparison to Wild-Type Phosphorylase *b*

wt phos. *b*[a,b,c]	-K[9]-R-K-Q-I-**S**-V-R-G-L[18]-
K11A	-K[9]-R-A-Q-I-**S**-V-R-G-L[18]-
K11E	-K[9]-R-E-Q-I-**S**-V-R-G-L[18]-
I13G	-K[9]-R-K-Q-G-**S**-V-R-G-L[18]-
R16A	-K[9]-R-K-Q-I-**S**-V-A-G-L[18]-
R16E	-K[9]-R-K-Q-I-**S**-V-E-G-L[18]-
wt phos. *b*	-T[38]-L-V-K-D-R-N-V-A-T[47]-
R43E	-T[38]-L-V-K-D-E-N-V-A-T[47]-
wt phos. *b*	-V[64]-G-R-W-I-R-T-Q-Q-H[73]-
R69K	-V[64]-G-R-W-I-K-T-Q-Q-H[73]-
R69E	-V[64]-G-R-W-I-E-T-Q-Q-H[73]-
wt phos. *b*	-P[497]-G-L-A-E-I-I-A-E[505]-
E501A	-P[497]-G-L-A-A-I-I-A-E[505]-

[a] Numbers next to residues denote the residue number in GP. [b] Boldface **S** represents the Ser 14 phosphorylation site. [c] Residues mutated are underlined.

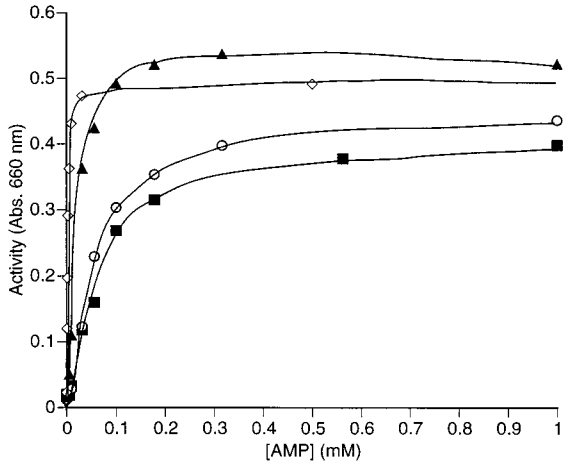

FIGURE 1: AMP activation curves for phos. *b* (■), I13G (○), R69E (▲), and R16E (◇). Activity of each mutant with increasing AMP concentration from 0 to 1 mM is reported as absorbance (Abs.) at 660 nm, after the methods of Krebs et al. (*22*). An Abs. of 0.3 is equivalent to 0.76 μmol of inorganic phosphate released by phosphorylase in the assay.

for 5 min prior to starting the reaction with γ(1−300), and a 15 μL aliquot was removed and spotted after 3 min of incubation with γ(1−300). Papers were washed and counted as described above.

RESULTS

Activity and AMP-Binding Characteristics. Phosphorylase mutants produced for these studies are shown in Table 1 and compared to the natural sequence in wild-type phos. *b*. Each mutant was assayed for its activity and for any changes in its AMP-binding characteristics in comparison to phos. *b*. AMP activation curves are shown for phos. *b* and three representative GP mutants in Figure 1. All mutants had levels of activity similar to that of phos. *b*. The maximum velocity of some of the mutants, however, was up to 20% higher than phos. *b* (data not shown). For the mutants illustrated in Figure 1, I13G most closely resembled phos. *b*. Both R69E and R16E, however, were activated at lower AMP concentrations, as can be seen by their curves being shifted to the left. From the AMP activation curves of each mutant, Hill plots (not shown) were produced to determine the cooperativity of AMP binding (n_H) and affinity for AMP (K_m) (Table 2).

Table 2: Hill Coefficients (n_H) and K_m of AMP Binding for phos. *b*, phos. *a*, and GP Mutants

GP variant	K_mAMP (μM)[a]	n_HAMP
phos. *b*	78.5 ± 10	1.39 ± 0.16
phos. *a*	1.5 (*1*)	1.0 (*6*)
K11A	36.4 ± 1.7	1.49 ± 0.01
K11E	14.3 ± 0.2	1.45 ± 0.045
I13G	71.9 ± 8.3	1.34 ± 0.014
R16A[b]	9.33	1.25
R16E	2.58 ± 0.5	1.29 ± 0.066
R43E	47.6 ± 1.9	1.28 ± 0.04
R69K	69.0 ± 10	1.3 ± 0.025
R69E	26.8 ± 3.5	1.8 ± 0.005
R43E/R69E	46.1 ± 8.9	1.23 ± 0.03
E501A[c]	17.8	1.55

[a] K_m is defined as the concentration of AMP at which GP achieves half-maximal velocity. [b] Four independent AMP activation curves were averaged together to produce one Hill plot. [c] Two independent AMP activation curves were averaged together to produce one Hill plot.

The AMP-binding characteristics of almost every mutant were different from that of phos. *b*. There was a large range of K_m values between the mutants. The K_mAMPs for I13G and R69K were similar to the value for phos. *b*. For K11A, R43E, R43E/R69E, and R69E, the K_ms have been slightly reduced from 1.65- to 3-fold. For K11E and E501A, the K_mAMPs were reduced 5.5- and 4.4-fold, respectively. The two mutants with the greatest affinity for AMP were R16A and R16E, with K_ms 8.4- and 30.4-fold reduced, respectively, compared to phos. *b*. The K_m values for these mutants were much closer to the K_mAMP of phos. *a* than of phos. *b* (Table 2). Another observation was that some of the mutants formed crystals in 40/5 buffer at low temperature, as phos. *a* does. Native phos. *b* does not crystallize under these conditions. R16A and R16E crystallized in 10 min on ice, or in 60 min at 4 °C, while K11E and E501A both required longer incubations (12−24 h) at 0 °C in order to form crystals (not shown). All of these crystals dissolved easily upon warming. It is notable that these four mutants also had the lowest K_m values for AMP.

The Hill coefficient for AMP denotes the level of cooperativity with which AMP binds to the enzyme. Phosphorylase *b* has been reported to have an n_H for AMP between 1.4 and 1.6 (*33*, *34*), indicating positive cooperativity of binding. Phosphorylase *a*, in contrast, has an n_H of 1.0, meaning no cooperativity of binding (*1*). In these studies we determined the n_H of phos. *b* to be 1.39, which agreed with the previous reports. None of the mutants exhibited drastically changed Hill coefficients. K11E, K11A, and E501A had a slightly higher n_H, while I13G, R69K, R16A, R16E, R43E, and R43E/R69E had a slightly lower n_H. Only R69E had a significantly increased n_H of 1.8.

Conformational Effects on Phosphorylation. Two mutants, R16A and R16E, were significantly changed from phos. *b* in their overall characteristics (Table 3). Their ability to crystallize and greater affinities for AMP indicated they were conformationally different from phos. *b* (T state) and may be closer to the conformation of phos. *a* (R state). We saw previously that these two mutants were phosphorylated poorly, exhibiting very high K_ms for phosphorylation by PhK γ(1−300) (*26*). This lack of phosphorylation could be due to either the change in primary structure or the change in conformation. To investigate this, we took advantage of GP's many effectors which change its conformational state.

Table 3: Summary: Comparison of Arg 16 Mutants to phos. *a* and phos. *b*

	phos. *b*	phos. *a*	R16A	R16E
K_mAMP (μM)	78.5	1.5 (*1*)	9.3	2.6
Hill coefficient, n_HAMP	1.39	1.0 (*6*)	1.25	1.29
activity without AMP	−	+	−	−
solubility in solution at 0 °C	+	−	−	−
sensitivity to G6P	+	−	−	−

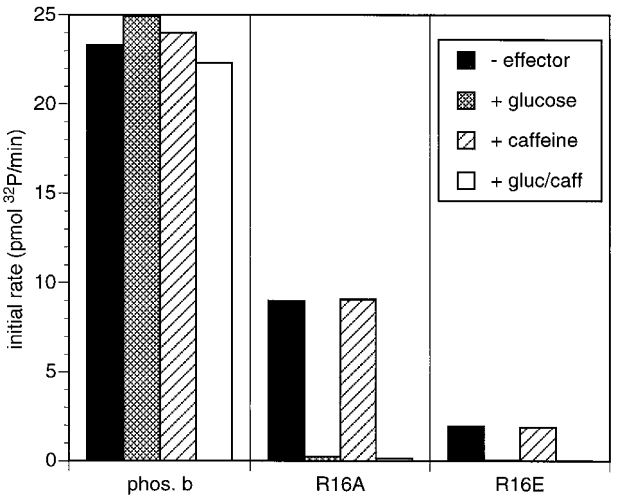

FIGURE 2: Phosphorylation of phos. *b*, R16A, and R16E in the presence of glucose (10 mM), caffeine (1 mM), glucose and caffeine (same concentrations), or no effector. The initial rate of phosphorylation by PhK γ(1−300) is reported as pmol of ^{32}P incorporated/min.

We phosphorylated phos. *b*, R16A, and R16E in the presence or absence of the T state effectors glucose and caffeine, which bind to and inhibit GP synergistically (Figure 2) (*20*). While neither effector, alone or together, had any effect on phos. *b* phosphorylation, glucose alone almost eliminated phosphorylation of R16A and R16E (Figure 2). We determined IC_{50} values for glucose inhibition of phosphorylation to be 330 μM for R16E, 500 μM for R16A, and greater than 1 M glucose for phos. *b* (Figure 3). We next investigated whether glucose and caffeine were still inhibitory to the activity of the Arg 16 mutants. Glucose or caffeine alone did slightly inhibit the activity of R16A and R16E, and inhibited even further when used together (Figure 4, panels B and C). This inhibition, however, could be overcome by AMP. In contrast, phos. *b* activity was completely inhibited by these effectors when used together, and this inhibition could not be overcome by up to 1 mM AMP (Figure 4A).

We then tested another T state effector (inhibitor) of phos. *b*, G6P, to see if it produced a similar effect. G6P, known to inhibit the phosphorylation of phos. *b* 20−30% (*23*), produced similar inhibition here (data not shown). Phosphorylation of R16A and R16E, however, was not inhibited by G6P (not shown). Because phos. *a* is not sensitive to G6P, this finding provided further support for the mutants being closer to the R state (Table 3). We next investigated the effect of an R state effector, AMP, on phosphorylation. AMP (300 μM) significantly inhibited phosphorylation of all the GP forms: 25% for phos. *b*, 75% for R16A, and 90% for R16E (Figure 5A). Because AMP binds to the mutants much more tightly than to phos. *b*, however, AMP was also used at the K_m for each GP form: 75 μM for phos. *b*, 10 μM for R16A, and 3 μM for R16E. At these concentrations, AMP had no effect on the phosphorylation of the mutants and only slightly inhibited phos. *b* phosphorylation (Figure 5B). A substrate (R state effector) of GP, G1P (50 mM), slightly inhibited phosphorylation of all the forms, and inhibited the mutants slightly more than phos. *b* (not shown). The K_m values for G1P binding to R16A and R16E were no different than phos. *b*, however (data not shown).

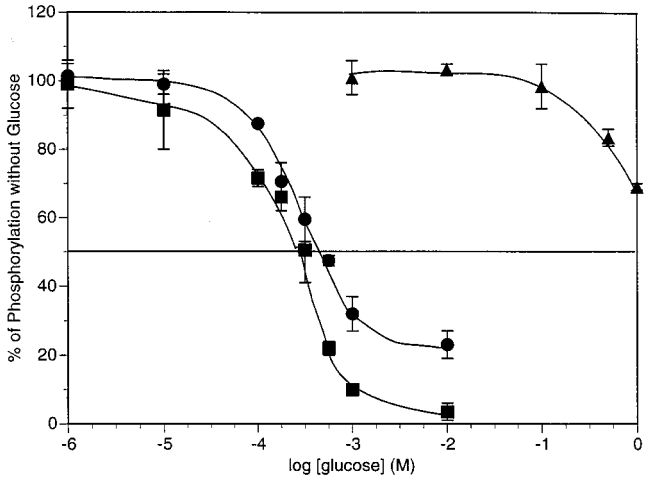

FIGURE 3: IC_{50} determination for glucose inhibition of phosphorylation of phos. *b* (▲), R16A (●), and R16E (■) by PhK γ(1−300). 2 mg/mL phosphorylase was preincubated with increasing concentrations of glucose for 5 min, followed by incubation with 0.2 μg/mL γ(1−300) for 3 min. Data are reported as the amount of ^{32}P incorporated into phosphorylase at each concentration of glucose as a percentage of that with zero glucose. From the graph, the concentrations of glucose at which phosphorylation is 50% inhibited (IC_{50}s) were determined to be >1 M for phos. *b*, 500 μM for R16A, and 330 μM for R16E.

Phosphorylase a *Activity of GP Mutants.* In testing the phosphorylation of the GP mutants by γ(1−300), progress of the reactions was measured both by the incorporation of ^{32}P from [γ-^{32}P]ATP and by the increase in phosphorylase activity without AMP. With six of the mutants (K11A, K11E, R16A, R16E, R69K, and E501A), the increase in activity reflected the incorporation of phosphate, even though some mutants were poorer substrates for γ(1−300) than others. This is best demonstrated by the mutants K11A and K11E in Figure 6. Figure 6A shows the increase in ^{32}P incorporation over time of incubation with γ(1−300), while similar curves are seen in Figure 6B for the increase in activity. Figure 6C compares the activity of each *b*-form plus AMP, and each *a*-form minus and plus AMP. These results demonstrate that all phosphorylated forms (*a*-forms) had similar levels of activity in the presence of 1 mM AMP, and that the phosphorylation- and AMP-induced activities were additive.

The same experiments with R69K and R69E showed that phosphorylation of phos. *b* and R69K were similar, as measured both by ^{32}P incorporation and by activation (data not shown). The phosphorylation and activation of R69E, however, were not equivalent. After 30 min, R69E showed roughly 60% as much phosphate incorporated as phos. *b*, but only 22% as much activity (data not shown). The phosphorylation of R69E, therefore, did not result in complete activation, as it did for phos. *b*. The activity of R69E in the presence of AMP was unaffected, but phosphorylation did not increase the AMP-induced activity (not shown). A more severe effect was seen with the mutant I13G.

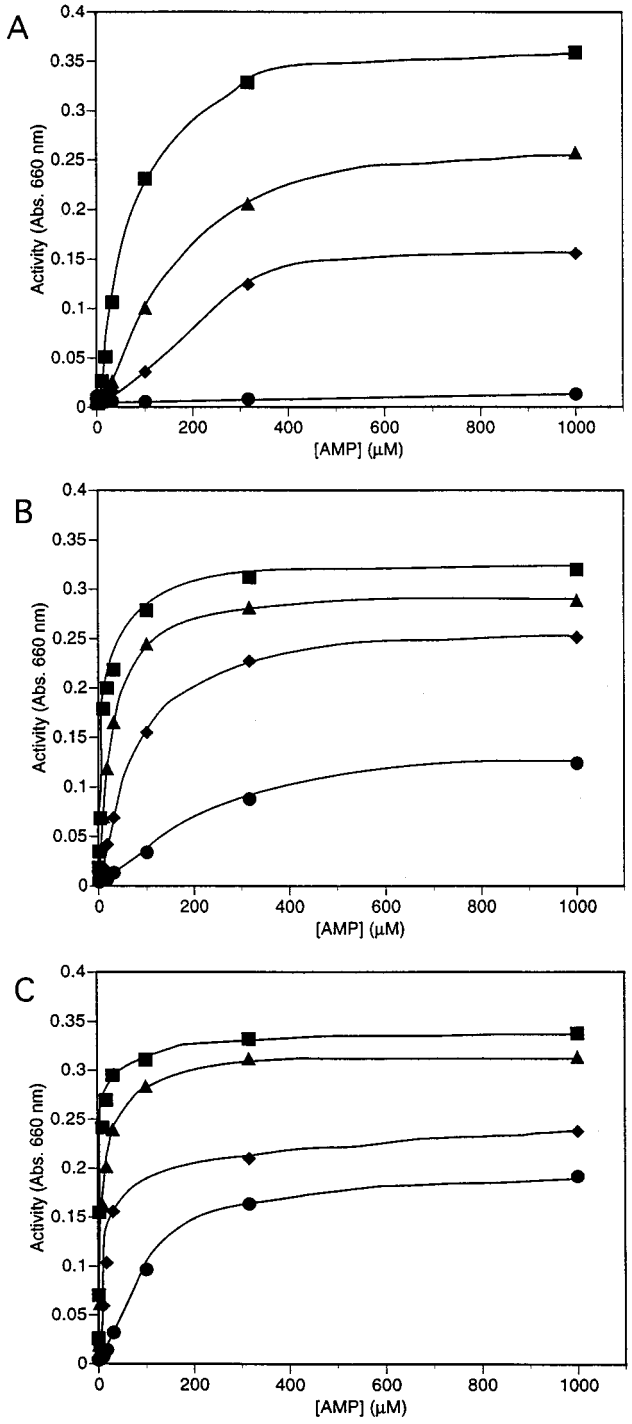

FIGURE 4: AMP activation curves for (A) phos. *b*, (B) R16A, and (C) R16E without effector (■) or with glucose (▲, 10 mM), caffeine (◆, 1 mM), or both (●, same concentrations). Activity was measured as in Figure 1 with increasing AMP concentration.

Figure 7A shows that I13G was phosphorylated at about 40% the rate of phos. *b*. This phospho-I13G, however, was completely inactive without AMP (Figure 7B). Like R69E, the activity of I13G-*a* plus AMP remained intact, but the phosphorylation did not add to the AMP-induced activity (Figure 7C). Mutants R43E and R43E/R69E displayed the same characteristic as I13G (Figure 8). While both were phosphorylated almost as well as phos. *b*, the phosphorylation did not result in activation of these mutants. There was a difference in AMP-induced activity, however. In R43E-*a*, although there was no activity without AMP, the serylphosphate did act additively with AMP once it was added, whereas with the double mutant, there was no additive effect of phosphorylation and AMP (Figure 8C).

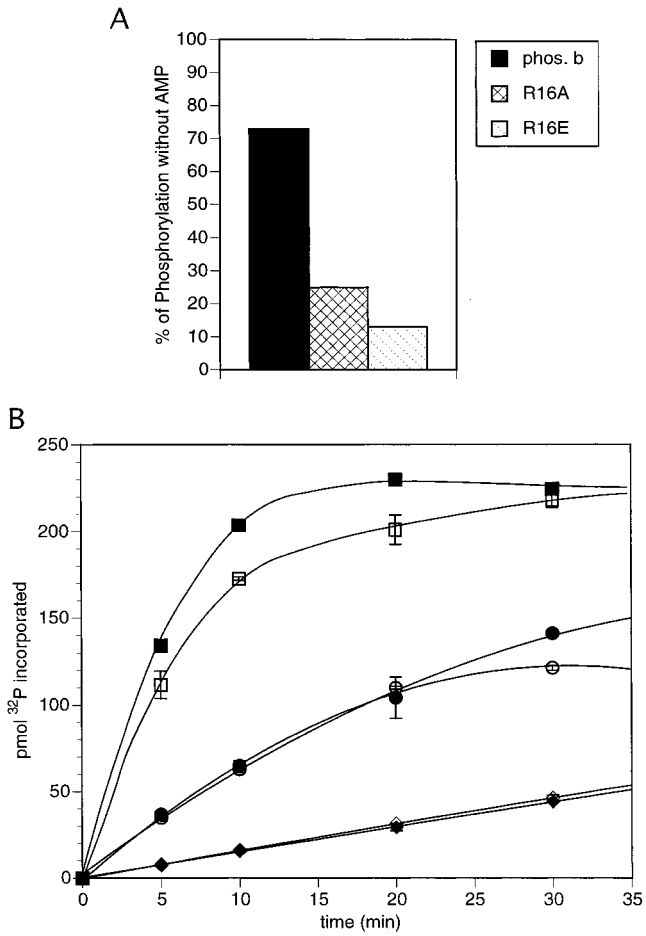

FIGURE 5: AMP inhibition of phosphorylation of phos. *b*, R16A, and R16E by $\gamma(1-300)$. (A) 300 μM AMP was added to assays of phosphorylation. Data are reported as the amount of phosphorylation of each GP form after 5 min as a percentage of that without AMP. (B) Time courses of phosphorylation of phos. *b* (■, □), R16A (●, ○), and R16E (◆, ◇) without (closed symbols) and with (open symbols) AMP at the concentration of the K_mAMP for each form. For phos. *b*, the AMP concentration was 75 μM, for R16A it was 10 μM, and for R16E it was 3 μM.

DISCUSSION

In our studies of glycogen phosphorylase, we have discovered several single residues in which mutations cause changes in its conformation or activity. This is most likely due to perturbations of the contacts each residue usually makes which contribute to conformation. In all of these mutants, we believe that the changes in conformation and activity can be attributed to a change in position and structure of the amino-terminal tail of GP. In this way, the amino terminus may act like a switch, whose structure and placement determine the structure of the core of GP.

The mutants R16A and R16E demonstrate that different electrostatic interactions of the N-terminus may change the conformation of GP. These mutants' characteristics, summarized in Table 3, indicate to us that their conformations are more like the R state than the T state of GP. To explore the possible reasons for this, we should consider the role of the N-terminus in wild-type GP. In phos. *a*, the N-terminus

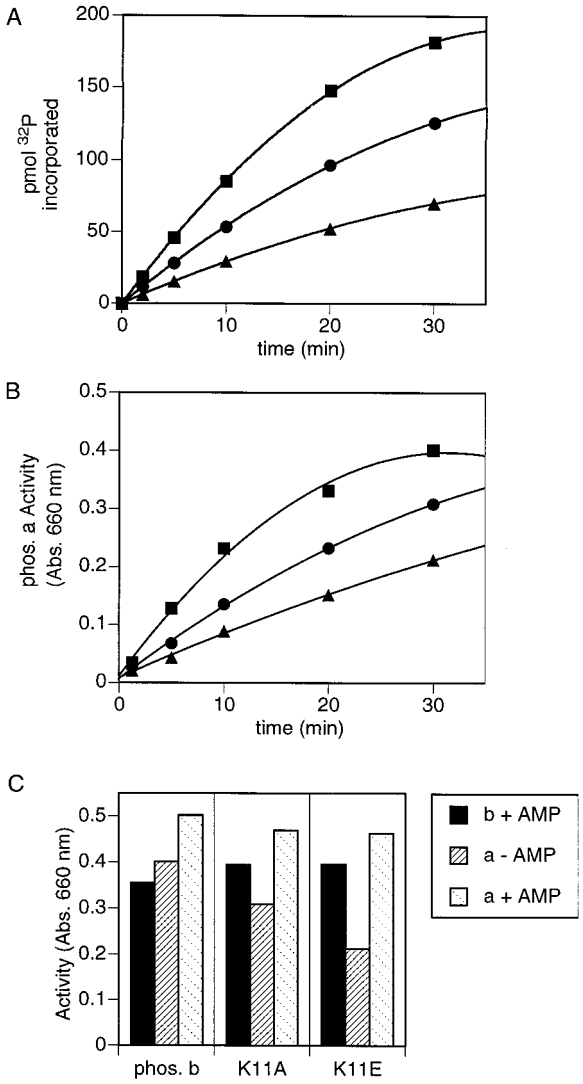

FIGURE 6: Time courses of phosphorylation of phos. *b* (■), K11A (●), and K11E (▲) by γ(1−300), measured by the increase in ^{32}P incorporation or phosphorylase activity. 2 mg/mL phosphorylase was incubated with 0.05 μg/mL γ(1−300). (A) Phosphorylation is reported as pmol of ^{32}P incorporated from [γ-^{32}P]ATP. (B) Aliquots were diluted to 20 μg/mL GP at each time point and assayed for phos. *a* activity (Abs. 660 nm, without AMP). (C) Phosphorylase activity of the 30 min time points of the experiments reported in (B): control reaction, not incubated with γ(1−300), plus AMP (b + AMP); reaction with γ(1−300), without AMP (a − AMP); reaction with γ(1−300), plus AMP (a + AMP).

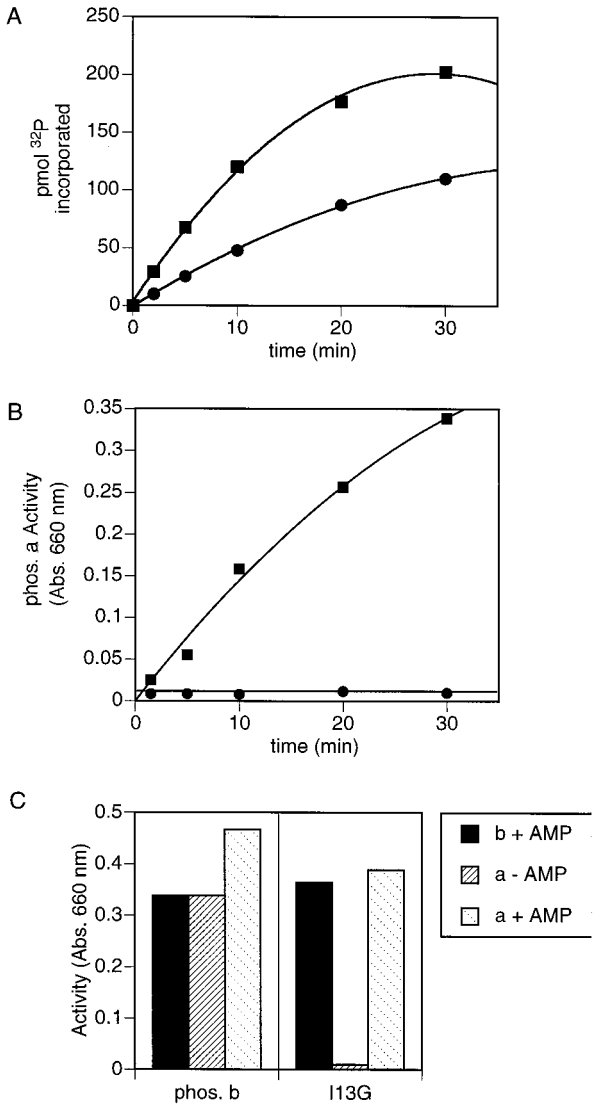

FIGURE 7: Phosphorylation of phos. *b* (■) and I13G (●), measured by (A) ^{32}P incorporation and (B) increase in phosphorylase activity, as in Figure 6. (C) Phosphorylase activity of the 30 min time points of the experiments reported in (B): control reaction, not incubated with γ(1−300), plus AMP (b + AMP); reaction with γ(1−300), without AMP (a − AMP); reaction with γ(1−300), plus AMP (a + AMP).

acts as a sort of R state effector, binding at the subunit interface. In phos. *b*, each positively charged N-terminus rests on the surface of its own monomer in a region of negative surface charge (*16*), but its functional role is not obvious. However, from many previous studies of phos. *b*′, which is missing the N-terminal tail, it is clear that the N-terminus contributes significantly to the structure and function of phos. *b* (*6*, *35*). Phosphorylase *b*′ has altered interactions with several effectors: it is not inhibited by G6P, it is not activated by sulfate, and it has low K_m for both AMP and inorganic phosphate (*36*, *37*). R16A and R16E have many of these same characteristics.

The loss of Arg 16, therefore, seems to have an effect similar to complete removal of the N-terminal tail, implying that in the mutants the N-terminus is no longer interacting with the surface. An explanation for this may be a change in electrostatic interactions. Phosphorylation of Ser 14 is thought to neutralize the basic charges across the N-terminus (*38*), which may normally electrostatically hinder formation of the 3_{10} helix. Phosphorylation, then, may not only allow helix formation, but also initiate release of the tail from the surface. Mutation of Arg 16 to Ala or Glu may have the same effect, neutralizing some of the interactions between the N-terminus and surface and allowing release of the tail from the surface. E501A and K11E, which also have lowered K_mAMP values and crystallize slowly, would also change these electrostatic interactions. They are not as different from phos. *b* as are the Arg 16 mutants, however. Change of Glu 501, in the region of negative surface charge, may only release the tail without affecting its structure. Lys 11, in contrast, is farther from the phosphorylation site than is Arg 16, so mutations there may be less effective in changing the N-terminal electrostatic interactions.

The changes made by the N-terminus in the mutants are significant, but are not enough to fully activate these forms of GP. Mutants of Ser 14 to Asp or Glu have some similar

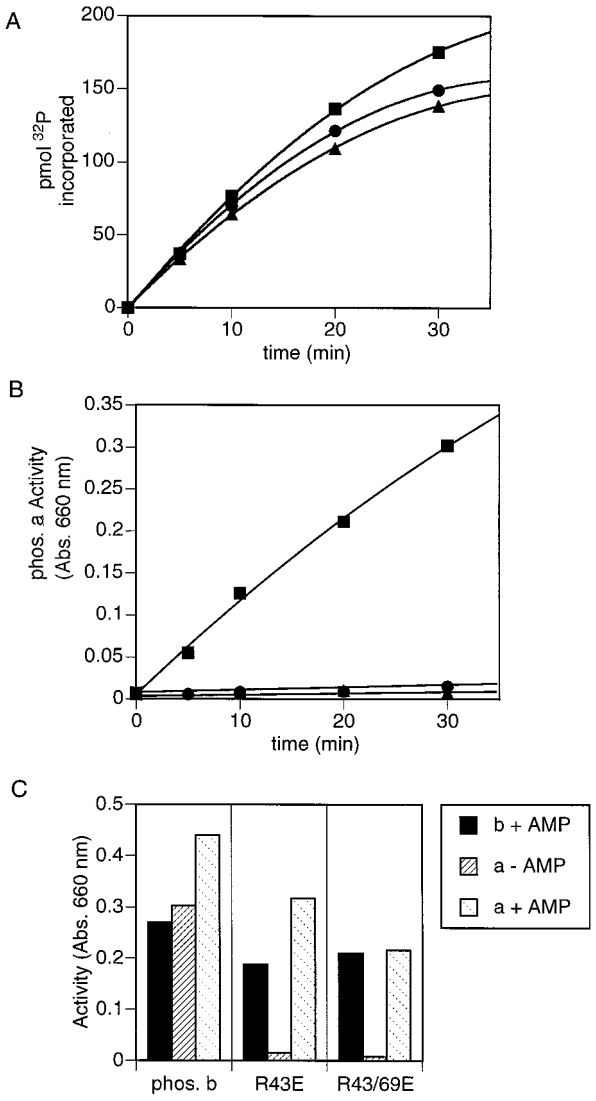

FIGURE 8: Phosphorylation of phos. *b* (■), R43E (●), and R43E/R69E (▲) measured by (A) ^{32}P incorporation and (B) increase in phosphorylase activity, as in Figure 6. (C) Phosphorylase activity of the 30 min time points of the experiments reported in (B): control reaction, not incubated with $\gamma(1-300)$, plus AMP (b + AMP); reaction with $\gamma(1-300)$, without AMP (a − AMP); reaction with $\gamma(1-300)$, plus AMP (a + AMP).

characteristics to these mutants, but are not fully activated either (*39*). Other studies indicate that a dianion may be necessary to produce the completely activated state (*16*, *40*). In any case, release of the N-terminus from the surface of GP, either by its complete removal or by altering its electrostatic interactions, seems to be sufficient to at least begin the shift to the R state. All of this implies that in phos. *b*, the N-terminus is not just passively resting on the surface, but is being "held" there by electrostatic interactions, and is responsible for keeping phos. *b* in an inactive conformation.

The results obtained with the Arg 16 mutants raise another question: is there a conformational state of GP which is preferentially recognized by PhK? This question has been explored for many years, but is difficult to answer because phos. *b* phosphorylation is slightly inhibited by most of its effectors, whether they are activators or inhibitors. Our studies show that the inhibitor caffeine has no effect on phosphorylation of phos. *b* or the mutants, while glucose severely reduces phosphorylation of the Arg 16 mutants. The IC_{50} for glucose inhibition of phos. *b* phosphorylation is 2000−3000 times greater than the values for R16A and R16E (Figure 3), implying that the apparent binding of glucose to the mutants is much tighter than to phos. *b*. The IC_{50} is not a measure of binding, however, so glucose may bind similarly to the mutants and phos. *b*, but this binding to R16A and R16E produces a different conformational "result" which is very poorly recognized by $\gamma(1-300)$.

A possible explanation for this begins with a comparison of RMGP to a more "primitive" enzyme. In yeast GP (YGP), the part of its N-terminus homologous to RMGP is not mobile, but is always at the subunit interface (*41*). In higher organisms, the N-terminus has evolved, with an Arg seemingly put especially at the P+2 site in mammalian GPs (Arg 16) to allow the N-terminus to move between the surface and the subunit interface in response to phosphorylation (personal communication, R. Fletterick). Since we have removed that Arg, we may have produced a sort of evolutionary "precursor GP", in which the equilibrium of the N-terminus does favor it being at the subunit interface as in YGP.

It has been shown that placement of the N-terminus at the subunit interface tends to make GP more stable, i.e., less conformational mobility (*42*). In wild-type GP, glucose also causes the molecule as a whole to become more stable, yet at the same time causes mobility of the N-terminus (*43*). It is possible that in R16A and R16E ("precursor GPs"), instead of causing mobility of the N-terminus, glucose further stabilizes the N-terminus so that it is less likely to be rejected from the interface. In other words, glucose may cause an ordering of the N-terminus in a way that makes the N-terminus inaccessible to the kinase.

Other GP effectors have different effects on phosphorylation of the mutants, which may also be due to changes in the N-terminus. The phos. *b* inhibitor G6P has no effect on R16A and -E phosphorylation, presumably because the mutants are in a phos. *a*-like conformation, which does not support binding of this ligand. The activator AMP inhibits phosphorylation of R16A and -E, but only at a concentration 30- or 100-fold above their respective K_mAMPs. It is possible that AMP saturation of these mutants promotes a phos. *a*-like ordering of the N-terminus which does not occur in wild-type GP, perhaps making the Ser 14 less accessible to the kinase. The question of whether PhK preferentially recognizes the R or T state of GP has still not been answered, then, and requires further study. In light of the data presented here, however, this question may not be as important. These mutants indicate that the accessibility of the N-terminus to the kinase may be more of a factor than the activation state of GP as a whole.

A different effect seen in some of the mutants of GP is phosphorylation without equivalent activation. R69E is activated at about one-third the rate of its phosphorylation, while I13G, R43E, and R43E/R69E cannot be activated at all by phosphorylation. The salt-bridges formed by Arg 43′ and Arg 69 to the serine phosphate group in phos. *a* appear to be required to hold phos. *a* in its active conformation. Replacement of either or both arginines with the acidic residue Glu may electrostatically repel the phosphate group and prevent the charge reversal mutants from becoming fully activated upon phosphorylation. It has been suggested that the underlying requirement for activation of phos. *a* is the

proper placement of the α2 helix and cap′ with respect to each other (*10*, *44*). Therefore, the Arg−phosphate interactions are likely to be the means to achieve this alignment. The two arginines do not seem to be completely equivalent, however, shown by the partial activity of R69E-*a*. In addition, R43E-*a*+AMP is more active than R43E-*b*+AMP (Figure 8C), while the double mutant has the same activity with AMP in either form, as does R69E (not shown). This also implies slightly different roles for Arg 43 and Arg 69. Arg 43 seems to be the more important residue for interaction with the serylphosphate, while Arg 69 by itself is able to support the additive effects of phosphorylation and AMP on GP activity.

I13G also has no activity without AMP after 30 min of phosphorylation (Figure 7). The role of Ile 13 in the structure of the N-terminus is not specifically known, other than its participation in the 3_{10} helix that forms after phosphorylation. Since the residue glycine in secondary structures is known to be a helix-breaker (*45*, *46*), the substitution of a Gly in this position may disrupt the 3_{10} helix. If this helix does not form properly, it is likely that the phosphoserine would not be positioned correctly to allow its interaction with the arginines, thus preventing activation of the *a*-form.

The data presented here are consistent with a model in which the position and structure of the N-terminus are critical for determining the conformation of GP. In phos. *b*, the highly basic amino-terminal tail is disordered and rests on an acidic patch on the surface of the protein. The positive charges on the N-terminus may hinder 3_{10} helix formation and interact with acidic surface residues to actively hold phos. *b* in the inactive conformation. Phosphorylation of Ser 14 then may neutralize these charges, allowing release of the N-terminus from the surface, formation of the 3_{10} helix, and movement of the tail to the subunit interface. Once at the subunit interface, the serylphosphate must make the necessary salt-bridge interactions with Arg 43′ and Arg 69 in order to achieve activation of phos. *a* in the absence of AMP.

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